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Multi Drug Resistance (MDR) is a phenomenon where tumor cells treated with one chemotherapeutic agent become resistant to many unrelated drugs. It is thought that most cancer MDR is due to the upregulation of drug efflux proteins, such as MDR1 or BCRP. We previously showed that ecto-phosphatase upregulation can lead to an MDR phenotype and ecto-phosphatase inhibition leads to breakdown of p-glycoprotein-mediated MDR implicating ecto-phosphatase activity as a second component of MDR. The experiments here, tested whether novel ecto-phosphatase inhibitors, added to the media, lowers the minimum inhibitory drug concentration (MIC) of non-drug resistant breast cancer cell lines or lowers the drug resistance of MDR lines. The hypothesis tested is that apyrase and other ecto-phosphatases are potential chemotherapeutic targets for the treatment of MDR cancers. These studies showed that there was no reduction in MIC for non drug resistant or drug resistant breast cancer cell lines. The long-term goal is to provide new tools to fight the growing problem of drug-resistant cancers by targeting drug efflux. Based on these exploratory experiments, it is concluded that ecto phosphatase activity alone is probably not a viable anti MDR target in breast cancer.

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INTRODUCTION

Multidrug Resistance (MDR) is a phenomenon whereby tumor cells (as well as fungal, plant and bacterial cells) treated with one chemotherapeutic agent become resistant to that and many other unrelated drugs (Ref 1). It is currently thought that most cancer MDR is due to the upregulation of drug efflux proteins, such as p-glycoprotein (MDR1) or Breast Cancer Related Protein (BCRP) but the exact mechanism remains controversial. Recently we showed that upregulation of ecto-phosphatase activity, particularly apyrase, leads to MDR and inhibition of this activity leads to breakdown of p-glycoprotein-mediated MDR (Ref. 3). This and other work implicates extracellular ATP concentration and ecto-phosphatase activity as a second component of the MDR phenomenon (Ref. 2). Via high-throughput screening of a diverse small molecule library, we isolated several novel, ecto-phosphatase inhibitors. In this project, the experiments proposed will used established cancer cell lines in vitro. The experiments tested whether the addition of ecto-phosphatase inhibitors to the media lowers the drug resistance of MDR cell lines or lowers the minimum inhibitory drug concentration of non-drug resistant lines. The goal of this work is to test the hypothesis that apyrase and other ectophosphatases are necessary accessory proteins to Multidrug Resistance efflux pumps in breast cancer cells and to test whether ecto-phosphatase activities are potential chemotherapeutic targets for the treatment of Multidrug Resistant cancers. The long-term goal is to provide clinicians with new tools to fight the growing problem of drug-resistant cancers by targeting, drug efflux, the most common mode of resistance.

FINAL REPORT BODY

In this project we proposed to test two predictions of our model for the function of ecto-phosphatase in drug efflux mechanisms in human breast cancer cell lines. In order to test this, we proposed two conceptually simple experiments using our novel inhibitors. In the first, we want to test whether addition of the ecto-phosphatase inhibitors allows us to lower the minimum inhibitory concentration of a drug in non-drug resistant cell lines. In the second, we want to test whether addition of the ecto-phosphatase inhibitors reverses the drug resistance of cells lines known to have a characterized Multi Drug resistance phenotype.

Prediction 1) Inhibiting ectophosphatases lowers the Minimum Inhibitory Concentration (MIC) of drugs necessary to kill non-drug resistant cell lines. We have found this to be true for yeast, plants, and bacteria (Ref. 3). Our novel inhibitors act as synergizers to lower the inhibitory dose needed for several unrelated classes of biocidal agents. The biocides include microtubulin inhibitors, such as oryzalin, closely related to anticancer drugs.

Prediction 2) Inhibiting ectophosphatases reverses MDR in characterized MDR cell lines. We have been able to reverse MDR in yeast, plants, and bacteria by inhibiting ectophosphatase activity. This was done genetically in yeast and epigenetically in plants and bacteria by using our novel inhibitors.

The above two predictions were tested in identical experiments with drug resistant and non-resistant cell lines.

Cell types used in study:
Human breast cancer cell lines:
MCF7, MCF7 Adriamycin resistant, MCF7 MDR1 overexpressing line, MCF7pcDNA3 (vector control line), and MCF7/BCRP clone 8--BCRP overexpressing line.
SW-13 and drug resistant variant.

IC50 studies were carried out to determine the concentration of inhibitor compounds which inhibited 50% of growth (IC50 value) and also to determine a concentration of inhibitor compound which gave less than 10% inhibition (to be used in synergizer studies). 4 cell lines and 10 inhibitor compounds were tested to obtain IC50 values. For all tests, cells were plated at 50,000 cells per well in sterile 96-well plates and cultured for 4 days at 37 ° C and 5% CO2. Media contained increasing concentrations of inhibitor compounds (0-90 μg/ml of each compound) with compounds initially dissolved in DMSO (DMSO was less than 0.5% of the media). Control wells (0 μg of inhibitor) had an equal volume of DMSO. All tests were done in triplicate to confirm results. After the 4 day period, cell viability was assayed using the MTT assay method. Briefly, 5 mg/ml MTT was dissolved in media and added to wells at 10% of the well volume (total volume of 150 μl). Plates were incubated for 3-4 hours. Media was then decanted and protein in wells was solubilized using 0.08 N HCl isopropanol. Plates were read spectrophotometrically on a plate reader at a wavelength of 570 nm.

For typical IC50 test data see Table 1 in appendix.

One or both of two compounds, the most potent of 2 classes (#1, a sulfonamide and #2, a N-phenyliminothiozole), were tested for the ability to synergize doxorubicin by increasing its cell growth inhibition at a given concentration. Doxorubicin was tested at concentrations from 0.0087-8.75 μg/ml. IC10 values for #1 and #2 were determined to be 0.117 μg/ml and

4.65 μg/ml respectively (using the methods described above). Both sensitive and resistant lines were tested.

Figure 1 (appendix) shows the results of growing non-resistant cell line, XXX, in increasing concentrations of Doxorubicin with and without the addition of inhibitor. As can be seen, the inhibitor had no affect on the ability of Dox to inhibit cell growth.

Figure 2 (appendix) shows the results of growing drug resistant cell line, XXX, in increasing concentrations of Doxorubicin with and without the addition of inhibitor. Although the IC50 value remained the same with and without Doxorubicin, there was an approximate 7-13% decrease in growth, as measured by absorbance at 570 nm, at concentrations of Dox between 0.0087-0.87 μg/ml when inhibitor #2 was added. This difference is not statistically significant (Student's T test). There was no difference in growth at any concentration when inhibitor #1 was added.

3) To support the above cell culture studies, we proposed a set of ancillary experiments to assess the ecto-phosphatase activities in MDR and non-MDR breast cancer cell lines. "We will separate and quantify the relative activities into apyrase, acid, alkaline, ecto-5'-nucleotidase, and other phosphatase. Using available antibodies to apyrase (CD39), MDR1, and drug sensitive and resistant breast cancer cells (such as MCF-7, MCF-7ADR, etc.) we will look for the co-occurrence of these proteins on the cell surface using immunocytochemistry and co-upregulation using FACS."

Because of the negative results with a large battery of resistance experiments, we greatly truncated this set of experiments. Using antibodies to CD39, we looked at the relative abundance of CD39 on the surface of resistant and non-drug resistant cell lines using confocal microscopy. We could detect no obvious quantitative difference between the sensitive and resistant lines.

KEY RESEARCH ACCOMPLISHMENTS

- Determined IC50 values for ecto-phosphatase inhibitors for sets of cancer cell lines.

- Tested the ability of ecto-phosphatase inhibitors to synergize the ability of anticancer drugs to inhibit non-drug resistant cell growth. Results negative.

- Tested the ability of ecto-phosphatase inhibitors to synergize the ability of anticancer drugs to inhibit multi drug resistant cell growth. Results negative.

REPORTABLE OUTCOMES

Several UT undergraduates received lab research experience through this grant. UT undergraduate, Monica Ghadia, received a UT-Austin Undergraduate Research Fellowship ($1000) based on this project.

PERSONELL RECEIVING PAY FROM THIS RESEARCH EFFORT.

Brian Windsor, Postdoctoral Fellow, Full time employee pay.
Alan Lloyd, PI, Associate Professor, One month's Summer salary.

CONCLUSIONS

There are two long-term goals of this project. This project is aimed at: 1. reducing the amount of chemotherapeutic agents required to kill a cancerous cell and 2. breaking down the multi drug resistance of cancerous cells that have developed MDR after chemotherapy. We have discovered an extracellular component of the MDR pump system, ecto-phosphatase, and isolated several novel inhibitors of this extracellular component. The experiments performed during this project were to designed to attack the ecto phosphatase by treating the cells with the novel inhibitors while simultaneously treating the cells with varying amounts of typical chemotherapeutic agents. The rationale was that disabling the external component of the major p-glycoprotein-based efflux system would allow more of the efflux pump substrate to remain in the cell. Prior to this project, we had demonstrated this to be true in yeast, bacteria, and plants and we had a small amount of preliminary data that it was true in mammalian cells.

The data generated in this project do not support AIM 1. No reduction in Minimum Inhibitory Concentration (MIC) was observed for non drug resistant breast cancer cell lines. This may be due to the existence of other efflux mechanisms that are not sensitive to external phosphatase activities. It also may be that the p-glycoprotein type efflux pumps are not significantly active in non MDR cell lines and that inhibition of these pumps has no detectable affect on retention of chemotherapeutic agents in these lines.

The data generated in this project indicate that adding ecto-phosphatase inhibitors does not significantly lower the cell growth inhibition activity of chemotherapeutic agent. Our preliminary data showed that an MDR cancer cell line was able to exclude a fluorescent molecule, calcein AM, and that adding an ecto-phosphatase inhibitor greatly increased the amount of calcein AM that was detected inside the cell. These were reproducible short-term uptake experiments. It may be that short-term inhibition of the efflux pump does not confer a decrease in MIC for these cells when the cells are subject to long-term exposure to the chemotherapeutic.

Another, very real possibility for the failure of these experiments is that the inhibitors we are using are not very effective on human ecto-phosphatases. They were isolated by their inhibition of potato apyrase because this apyrase is commercially available (Sigma). The human apyrase (CD39) is reported to be a very abundant ecto-phosphatase and it may be that our inhibitors are not very effective on CD39 or that there are other more important ecto-phosphatases. In that case, we would need to screen for new inhibitors.

These experiments were meant to be exploratory in nature and we feel we have given the model a robust test under the limits of these in in vitro conditions. We have to ask the question "So what next?". Based on these less-than-spectacular results, we do not intend to pursue this line of research in this way. As stated in the original proposal, we have shown that these inhibitors work to lower MICs in yeast, plants, and bacteria, in both "normal" and MDR equivalent lines. In parallel to the experiments discussed here, we have continued to study the resistance phenomenon in these other kingdoms. Those experiments have been far more successful and we are pursuing these lines of study.

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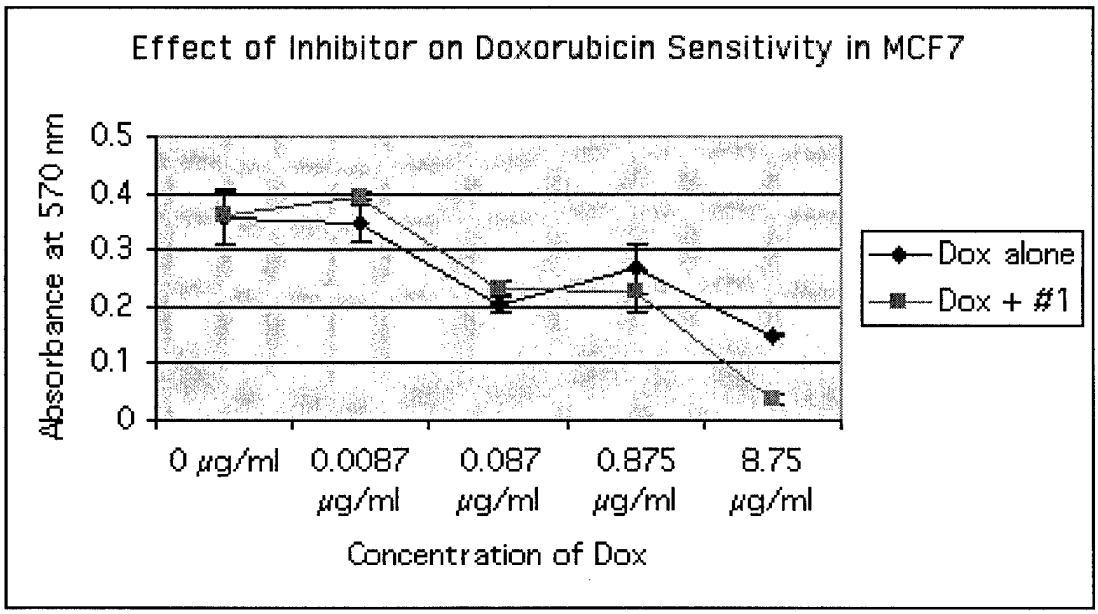
Appendix

Table 1 Typical IC50 test data (in this case using SW-13 cells):
Inhibitor compound I.D.#5, a napthylacetyl hydrazone class compound, IC50 was determined to be between 40 and 50 µg/ml.

µg/ml	0	10	20	30	40	50	60	70	80	90
λ at 570 nm	.824	.635	.522	.441	.443	.295	.317	.310	.449	.264

Figure 1. Non-drug resistant cell line, MCF7.



Appendix

Figure 2. Drug resistant cell line MCF7 MDR1.